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(54) Title: DIMERIC IL-4 INHIBITORS

(57) Abstract

The present invention relates to dimeric compounds containing IL-4 receptors. The dimeric compounds can be homodimers in which two IL-4 receptors are bound by a polymeric spacer and heterodimers in which an Il-4 receptor is linked by a polymeric spacer to an IL-2 receptor gamma chain. The invention also provides methods of making and using such compounds as well as pharmaceutical compositions containing the compounds.

DIMERIC IL-4 INHIBITORS

Field of the Invention

This invention relates to compounds that bind interleukin-4 (IL-4) and, more particularly, to dimeric compounds that are effective inhibitors of IL-4.

Background of the Invention

The therapeutic utility of an IL-4 inhibitor stems from the central role of IL-4 in the development and maintenance of primary and secondary immunoglobulin E (IgE) responses and in the development of T cell helper type 2 (Th2)-associated immune responses. The critical role of IL-4 in these immune responses has been documented through use of anti-IL-4 monoclonals and IL-4 deficient mice.

For example, elevated IgE levels are associated with immediate hypersensitivity diseases such as asthma, allergic rhinitis, atopic dermatitis and eczema, among others (Geha, Current Opinion in Immunology, vol. 5, pg. 935-936, 1993). IgE binds to specific receptors on mast cells and basophils. When cross-linked by antigen, the aggregated IgE causes mast cell/basophil degranulation, histamine release and cytokine synthesis. IL-4 has been shown to induce IgE synthesis by peripheral blood mononuclear cells in vitro (Garrone et al., Eur. J. Immunology, vol. 21, 1365-1369, 1991). Neutralizing monoclonal antibodies to mouse IL-4 have been shown to inhibit primary and secondary IgE responses in vivo without significantly affecting immunoglobulin G (IgG) responses (Finkelman et al., J. Immunology, vol. 141, pg. 2335-2341, 1988). Neutralizing monclonals to mouse IL-4 also have been shown to significantly reduce circulating IgE levels in mice with pre-existing hypersensitivity conditions (Finkelman et al., J. Immunology, vol. 141, pg. 2335-2341, 1988). Transgenic mice in which the IL-4 gene has been disrupted are unable to make IgE antibodies in response to allergen challenge (Bleuthmann et al., J. Cellular Biochemistry, vol. 0, Supplement 17B, pg. 120, 1993), treatment with polyclonal B-cell activators such as anti-IgD or when infected by nematodes (Kopf et al., Nature, vol. 352, pg. 245-248, 1993). In addition, IL-4 deficient mice show reduced

peribronchial inflammation in a model of antigen-induced airway inflammation, confirming the role of IL-4 and IgE antibodies in disease pathogenesis (Cuvelier et al., J. Cellular Biochemistry, vol. 0, Supplement 17B, pg. 87, 1993).

With regard to the role of IL-4 in T cell development, IL-4 and gamma interferon have been shown to play critical roles in determining whether immature Th0 cells develop into Th1 or Th2 type mature T cells (Maggi et al., J. Immunology, vol. 148, pg. 2142-2147, 1992). The effects of these cytokines appear to be reciprocal. Th1-type responses are associated with cellular immune responses, whereas Th2-type responses are associated with humoral immune responses. IL-4 has been shown to induce Th0 cells to become Th2-like. Similarly, antibodies to IL-4 have been shown to inhibit development of Th2 cells and allow development of Th1 cells. Th2 cells characteristically secrete IL-4, Il-5 and IL-10 upon activation. IL-10 acts to inhibit development of Th1 cells and down regulates the antimicrobial activities of monocytes and macrophages. In contrast, gamma interferon induces Th0 cells to become Th1-like. Th1 cells are characterized by the secretion of IL-2 and gamma interferon when activated. Gamma interferon also inhibits development of Th2 cells. Thus, once a particular type of immune response (Th1 or Th2) becomes established it tends to propagate itself through secretion of cytokines such as IL-4 and gamma interferon.

The ability to modulate whether a patient develops a Th1- or a Th2- associated T cell immune response could be useful clinically for at least two reasons. First, Th2-type T cells secrete cytokines such as IL-5 that are implicated in pathological processes such as eosinophilia in asthma patients. Inhibiting development of Th2 T cells could reduce IL-5 levels in patients and prevent development of the pathological consequences of eosinophilia such as airway damage and hypersensitivity. As reported in Cuvelier et al., J. Cellular Biochemistry, 0(17B):87 (1993) and Kopf et al., Nature, 362:245-248 (1993), IL-4 deficient mice do not develop eosinophilia and exhibit reduced airway inflammation in a model of antigen induced airway inflammation.

Second, altering a patient's immune response from a Th2 to a Th1 response could be beneficial clinically because animal studies have shown that persistent

infections by yeast and intracellular parasites such as Leishmania result from the susceptible host developing a Th2-associated immune response rather than a Th1-associated immune response. Antibodies to IL-4 prevent development of a Th2-associated response in susceptible animals and allows them to be "cured" of their persistent infections (Romani et. al., Infection and Immunity, vol. 59, pg. 4647-4654, 1991; Romani et al., J. Exp. Med., vol. 176, pg. 19-25, 1992; Sadick et al., J. Exp. Med., vol. 171, pg. 115-127, 1990). Soluble IL-4R also prevented development of a Th2-associated response in susceptible animals as reported in Gessner et al., Infection & Immunity, 62:4112-4117 (1994). The immunity conferred by anti-IL-4 therapy appears to be sustainable and to involve development of a Th1 response to the pathogen.

Some reports suggest that infection by the HIV virus results in a shift from a Th1 to a predominantly Th2 immune response. The inability to mount a Th1-associated immune response has been suggested to be a reason for the inability of certain patients to rid themselves of the AIDS virus and associated opportunistic infections (Clerici and Shearer, Immunology Today, vol. 14, pg. 107-111, 1993; Mosmann, T.R., Science, vol. 265, p. 193-194, 1994). In a subset of AIDS patients, treatment of peripheral blood lymphocytes obtained from the AIDS patients with anti-IL-4 antibodies in vitro restored the ability of these cells to proliferate in response to challenge with influenza A virus. This result suggests that anti-IL-4 therapies might be useful in restoring proper immune responses in some AIDS patients (Clerici and Shearer, J. Cell. Biochemistry, 0(17B):88 (1993)).

Accordingly, a need exists for IL-4 inhibitors to reduce IgE levels and alleviating symptoms in immediate hypersensitivity diseases such as allergic rhinitis, asthma, atopic dermatitis and eczema, among others. An IL-4 inhibitor is also needed to alter a patient's immune response from a Th2- to a Th1-associated response, which may be beneficial to patients suffering from recurrent infections caused by yeast, intracellular parasites and viruses. The present invention satisfies these needs and provides related advantages as well.

Summary of the Invention

The present invention relates to dimeric compounds capable of binding and inhibiting the activity of IL-4. The dimer compounds have the formula R_1-X-R_2, wherein R_1 is an IL-4 receptor; R_2 is an IL-4 receptor or an IL-2Rγ_c; and X is a polymeric spacer. The receptors comprise the extracellular domains of the naturally-occurring IL-4R and IL-2Rγ_c and active fragments thereof. X is polyethylene glycol having a molecular weight in the range of about 1 - 20 kD, preferably up to about 8 kD, and more preferably up to about 3.4 kD. The dimeric compounds can be homodimers when R_1 and R_2 are IL-4 receptors and heterodimers when R_1 is an IL-4 receptor and R_2 is an IL-2Rγ_c.

The invention further provides methods for making the dimeric compounds. The methods can be accomplished by recombinant DNA technology.

In a further embodiment, the invention relates to methods of treating an IL-4 mediated disease by administering a therapeutically effective amount of the dimeric compounds of the invention. The dimeric compounds can mediate the deleterious increase in IgE production and inhibit the development of Th2 T cells associated with certain IL-4 mediated diseases. Examples of IL-4 mediated diseases include allergic rhinitis, asthma, atopic dermatitis, eczema, infections caused by yeast, intracellular parasites or viruses, such as HIV. Pharmaceutical compositions are also provided for the use in the treatment of IL-4 mediated diseases.

The present invention also relates to the use of the dimeric compounds as diagnostic reagents and calibration standards for detecting or quantifying IL-4 in a sample. The methods include: (a) contacting the dimeric compounds of the invention with the sample; (b) allowing the dimeric compounds to bind to IL-4; and (c) detecting or quantifying IL-4 bound to the dimeric compounds.

The dimeric compounds can also be used to purify IL-4 from a sample. In these methods, the sample is first contacted with the dimeric compounds and allowed to bind. The bound IL-4 is then dissociated from the dimeric compounds and collected as purified IL-4.

Detailed Description of the Invention

The present invention provides dimeric compounds that are better or equal inhibitors of IL-4 than soluble IL-4 receptors. More particularly, the dimeric compounds have the formula R_1-X-R_2, wherein R_1 is an IL-4 receptor (IL-4R); R_2 is either an IL-4R or IL-2 receptor gamma chain (IL-2Rγ_c); and X is a polymeric spacer, also referred to herein as a cross-linker. Accordingly, the dimeric compounds of the present invention contain at least one biologically active IL-4 receptor.

As used herein, "dimeric compounds" refer to compounds containing two active moieties that can be the same or different. "Homodimeric compounds" refer to compounds containing the same biologically active moieties, such as two IL-4 receptors. "Heterodimeric compounds" refer to compounds containing two different active moieties, such as an IL-4 receptor and IL-2Rγ_c to form the IL-4 binding compounds of the present invention.

Murine and human IL-4 receptors have been cloned as described in Mosley et al., Cell, 59:335-348 (1989) and Galizzi et al., Int. Immunol., 2:669-675 (1990). A soluble form of the IL-4 receptor (sIL-4R) has been detected in mouse biological fluids, indicating that IL-4 receptors may be shed naturally from cells. A cDNA encoding a naturally-occurring soluble form of the mouse IL-4 receptor has been described in Mosley et al., Cell, 59:335-348 (1989). The cDNA encodes a protein that is missing the transmembrane and cytoplasmic domains of the receptor. In addition, the last 6 amino acids of the extracellular domain have been replaced by alternative amino acids. The cDNA appears to have been generated by alternative mRNA splicing. The DNA sequence of the human IL-4 receptor is provided in Galizzi et al., supra.

Studies have shown that the mouse sIL-4R, either the recombinant protein encoded by the alternatively spliced cDNA or the naturally occurring protein purified from biological fluids, is capable of binding IL-4 and inhibiting its actions on cells in vitro and in vivo (Mosley et al., Cell, 59:335-348 (1989; Fernandez-Botran & Vitetta, J. Exp. Med., 174:673-681 (1991)). However, the affinity of the sIL-4R for IL-4 is less than that of the IL-4R on cells. The reason for this difference appears to be a faster dissociation rate of IL-4 from the sIL-4R at 37°C relative to the cell bound IL-4R

(Fernandez-Botran and Vitetta, J. Exp. Med., 174:673-681 (1991). At 37°C, the half-life of the IL-4:sIL-4R complex was estimated to be 2.5 minutes compared to 69 minutes for the half-life of the IL-4:cell bound IL-4 receptor. The association rate of IL-4 with the sIL-4R appears to be faster than that of IL-4 with the cell bound IL-4R. The difference in association and dissociation rates between the sIL-4R and cell bound IL-4R for IL-4 results in a 3-fold difference in k_d values determined in equilibrium binding studies (Fernandez-Botran and Vitetta, J. Exp. Med., 174:673-681 (1991).

In vitro studies with a soluble form of the human IL-4R indicate that it also is capable of binding and neutralizing the activity of IL-4 on cells (Garrone et al., Eur. J. Immunol., 21:1365-1369 (1991). The human sIL-4R used was a truncated form of the cell bound receptor (missing the transmembrane and cytoplasmic domains) secreted from a mammalian cell line (COS cells). The affinity of the soluble human receptor for IL-4 has not been reported.

Recent studies suggest that a component of the IL-2 receptor known as the IL-2R gamma chain (IL-2Rγ_c) may be required for IL-4 signaling via the IL-4 receptor (Russell et al., Science, vol. 262, pg. 1880, 1993; Kondo et al., Science, vol. 262, 1874-1875, 1993). The IL-2R gamma chain also has been implicated in IL-7 signaling via the IL-7 receptor (Noguchi et al., Science, vol. 262, pg. 1877-1880, 1993). Equilibrium binding studies indicate that co-transfection of COS cells with the IL-4R and the IL-2R gamma chains leads to an increased affinity (3 to 4 fold) of the cell-bound IL-4R for IL-4. Russell et al., Science, 262:1880 (1993). In addition, a monoclonal antibody to the gamma chain, TUGm2, suppressed the high affinity binding of IL-4 to the CTLL-2 mouse T-cell ine by 6 to 7 fold (Kondo et al., Science, vol. 262, pg. 1874-1877, 1993). In studies related to the present invention, it was found that IL-2Rγ_c modulates the affinity of the IL-4R for 1L-4 by increasing IL-4R's high affinity interaction with IL-4. The DNA sequence for human IL-2Rγ_c has been previously published.

It was surprising to discover that the heterodimeric compounds of the present invention could be prepared that have higher affinities for IL-4 than the soluble IL-4 receptors as shown by an increase in bioactivity. It has also been found in the context

of the heterodimeric compounds (IL-4R:IL-2Rγ_c), that the IL-2Rγ_c portion provides increased bioactivity compared to mono-pegylated IL-4R or unpegylated IL-4R as shown by a decrease in dissociation rates reported in Example 8. It was unexpected that the IL-2Rγ_c would increase affinity by about 30 fold in light of Russell et al., Science, 262:1880 (1993); Kondo et al., Science, 262:1874-1875 (1993); and Noguchi et al., Science, 262:1877-1880 (1993), which only showed an increase in affinity from 3 to 7 fold. The length of the polymeric spacer also had an affect on the bioactivity of the dimeric compounds of the present invention. It was unexpectedly found that the shorter the length of the polymeric spacers, the greater the affinity of the dimeric compounds for IL-4. For example, a 20kD PEG heterodimer had slightly better IC_{50} values (1-2 fold) than the soluble IL-4R, while a 3.4kD PEG heterodimer had substantially increased IC_{50} values (up to 38-fold). It is believed that the shorter spacer length facilitates a higher local effective concentration of the stabilizing IL-2Rγ_c subunit.

As indicated above, the dimeric compounds of the present invention contain an IL-4 receptor that is purified from a naturally-occurring source or by other means known in the art, such as by chemcial synthesis or recombinant DNA technology as described in EP Patent Application No. 0 367 566 A1, published on May 9, 1990, entitled "Interleukin-4 Receptors," which is incorporated herein by reference. Similarly, IL-2Rγ_c can also be obtained by various means known in the art.

The preferred method for the production of the proteins is by recombinantly expressing the gene coding for the IL-4 receptor and IL-2Rγ_c. The genes coding for the desired proteins can be expressed in a variety of expression systems, including mammalian, yeast, insect and bacterial systems such as baculovirus and *E.coli*. The Examples below provide particularly useful methods of obtaining the IL-4 receptor and IL-2Rγ_c for use in the preparation of the compounds of the present invention.

In a preferred embodiment of the invention, the IL-4 receptor and IL-2Rγ_c are human. In this context, the IL-4 receptor (IL-4R) is the soluble human IL-4 receptor extracellular domain that contains 207 amino acids (1-207), while the IL-2Rγ_c refers to the soluble human IL-2Rγ_c extracellular domain that contains 254 amino acids (1-

254). As used herein, the terms "IL-4 receptor" or "IL-2Rγ_c" also include biologically active fragments of these extracellular domains. One skilled in the art can readily determine such active fragments without undue experimentation by testing the fragments in bioassays, such as the TF-1 bioassay set forth in Example 7 below.

However, to the extent that there is sufficient homology between animal DNA and peptide sequences to the human forms, they would also be included within the scope of this invention. In addition, alterations in the DNA or amino acid sequence of the human IL-4 receptor or IL-2Rγ_c, such as substitutions, additions or deletions that do not substantially affect the desired activity of the expressed protein, are also included within the definitions of an IL-4 receptor and IL-2Rγ_c. As used herein, "IL-2Rγ_c-FLAG" refers to the 254 amino acid extracellular domain of IL-2Rγ_c plus an added cysteine at the C-terminal end, plus GG and the FLAG™ octapeptide sequence DYKDDDDK (SEQ.ID.NO. 1) obtained from IBI, Eastman Kodak Co. (New Haven, CT). The altered gene can be created either by standard site specific mutagenesis procedures or by the construction of the altered gene by standard gene synthesis procedures. These techniques are well known to those skilled in the art.

Polymeric spacers useful in the present invention include, for example, polyethylene glycol, monomethoxy polyethylene glycol, polypropylene glycol, polyoxyethylated glycerol, dihydroxy polyethylene glycol, dextran, colonic acids, carbohydrate polymers, amino acid polymers or biotin derivatives. Other useful polymeric spacers include those known in the art such as peptide linkers, including F_c portions of immunoglobulins and inert sequences of amino acids, and nucleic acid linkers.

Polyethylene glycols (PEGs) are particularly useful as spacers in the present invention. PEGs are ideal for modifying proteins due to their proven non-toxic and inert properties. In addition, the modification of polypeptides with PEG (referred to as "PEGylation") serves to improve desirable pharmacokinetic properties in at least one of the following ways:

(1) increasing the apparent molecular weight of the native polypeptide(s) and, therefore, reducing the clearance rate following subcutaneous or systemic administration;

(2) increasing the solubility of the native polypeptide in aqueous solutions; or

(3) reducing the antigenicity of the native polypeptide.

Accordingly, the dimeric compounds of the present invention will have a therapeutic value above the soluble IL-4 receptor due to increased pharmacokinetic properties. In addition, PEG also facilitates the crosslinking of two receptor subunits to increase bioactivity for IL-4.

Useful PEGs of the present invention are those having molecular weights of up to 20,000, preferably those having molecular weights of 8,000 or less, and more preferably molecular weights of 3400 and less. The approximate molecular weight of a polymeric unit is given in subscripts. In the studies reported in the Examples below, the dimeric compounds containing various sizes of PEG were found to be about 1.3- to about 40-fold more effective in inhibiting the activity of IL-4 compared to wild-type soluble IL-4 receptor depending on the size of the PEG used as the spacer, with PEG_{3400} more effective than PEG_{8000}, which in turn was more effective than $PEG_{20,000}$ as reported in the Examples below. The results, therefore, showed a trend toward improved bioactivity with decreasing PEG size.

A functional or reactive group attached to the non-peptidic spacer is referred to herein as the activating group or linker. Activating groups include the maleimide group, sulfhydryl group, thiol, triflate, tresylate, aziridine, oxirane, 5-pyridyl, NHS esters and vinyl-sulfones as described in U.S. Patent Application No. 08/259,413, filed June 14, 1994, incorporated herein by reference.

The PEGylated dimeric compounds can be prepared according to the procedures set forth in WO 92/16221, published on October 1, 1992, specifically incorporated herein by reference, or as set forth in the Examples below.

The dimeric compounds of the present invention have a number of *in vitro* and *in vivo* uses. For example, the dimeric compounds can be used to affinity purify IL-4

from a number of sources, including serum from patients, cell culture supernatants or recombinantly produced IL-4, according to purification procedures. Briefly, methods of purifying IL-4 from a sample are accomplished by: (a) contacting a dimeric compound of the present invention with the sample; (b) allowing th dimeric compound to bind to IL-4; (c) dissociating the IL-4 from the dimeric compound; and (d) collecting the dissociated IL-4. Conventional affinity purification methods can be used in which the dimeric compounds of the present invention are attached to resin, bead or other conventional matrix and placed in a column or other receptacle. The sample is then loaded and eluted with an appropriate solution that can readily be determined by those skilled in the art to obtain purified IL-4.

The dimeric compounds of the present invention can also be used as diagnostic reagents to detect or quantify IL-4 according to diagnostic methods well known in the art. Generally, methods of detecting or quantifying IL-4 in a sample is accomplished by (a) contacting a dimeric compound with the sample suspected of containing IL-4; (b) allowing the dimeric compound to bind to IL-4; and (c) detecting or quantifying IL-4 bound to said dimeric compound.

The dimeric compounds of the present invention can additionally be used as calibration standards in a double determinant sandwich ELISA or in a solid phase binding assay. Such assays are well known in the art.

Another application for the use of the dimeric compounds is to inhibit IL-4 binding to target cells and thereby neutralize IL-4 bioactivity. In published studies, neutralizing antibodies were shown to be effective in inhibiting IL-4. In other studies, it was shown that sIL-4R ws more effective in neutralizing IL-4 than these known antibodies. In the studies reported herein, the dimeric compounds of the present invention were shown to be as effective or more effective in neutralizing IL-4 bioactivity than sIL-4R.

The dimeric compounds can also be used as immunogens to produce polyclonal and monoclonal antibodies according to procedures well known in the art and as described, for example, in Harlow & Lane, Antibodies: A Laboratory Manual (1988), incorporated herein by reference. Such antibodies can, in turn, be used to detect IL-4

receptors or receptor complexes on the cell surface of T cells for *in vitro* and *in vivo* uses such as imaging or inhibiting the binding of IL-4 to the receptors according to procedures known in the art.

In another embodiment, the dimeric compounds of the present invention can also be used to treat patients having an IL-4 mediated disease. The dimeric compounds can be used to inhibit deleterious or pathological IgE production as well as the undesirable overproduction of Th2 T cells associated with IL-4 mediated diseases. Such IL-4 mediated diseases include, for example, allergic rhinitis, asthma, atopic dermatitis and eczema. In addition, the compounds can be used to treat patients having recurrent infections caused by yeast, intracellular parasites and viruses, including HIV.

For these therapeutic uses, the dimeric compounds can be formulated into a pharmaceutically-acceptable carrier to form the pharmaceutical compositions of the present invention. The term "pharmaceutically acceptable carrier" as used herein means a non-toxic, generally inert vehicle for the active ingredient, which does not adversely affect the ingredient or the patient to whom the composition is administered. Suitable vehicles or carriers can be found in standard pharmaceutical texts, for example, in Remington's Pharmaceutical Sciences, 16th ed., Mack Publishing Co., Easton, PA (1980), incorporated herein by reference. Such carriers include, for example, aqueous solutions such as bicarbonate buffers, phosphate buffers, Ringer's solution and physiological saline. In addition, the carrier can contain other pharmaceutically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation.

The pharmaceutical compositions can be prepared by methods known in the art, including, by way of an example, the simple mixing of reagents. Those skilled in the art will know that the choice of the pharmaceutical carrier and the appropriate preparation of the composition depend on the intended use and mode of administration.

In one embodiment, it is envisioned that the carrier and the dimeric compounds constitute a physiologically-compatible, slow-release formulation. The primary solvent in such a carrier can be either aqueous or non-aqueous in nature. In addition, the carrier can contain other pharmacologically-acceptable excipients for modifying or maintaining the pH, osmolarity, viscosity, clarity, color, sterility, stability, rate of dissolution, or odor of the formulation. Similarly, the carrier can contain still other pharmacologically-acceptable excipients for modifying or maintaining the stability, rate of dissolution, release, or absorption of the dimeric compounds. Such excipients are those substances usually and customarily employed to formulate dosages for parenteral administration in either unit dose or multi-dose form.

Once the pharmaceutical composition has been formulated, it can be stored in sterile vials as a solution, suspension, gel, emulsion, solid, or dehydrated or lyophilized powder. Such formulations can be stored either in a ready to use form or requiring reconstitution immediately prior to administration. The preferred storage of such formulations is at temperatures at least as low as 4°C and preferably at -70°C. It is also preferred that such formulations containing the dimeric compounds are stored and administered at or near physiological pH. It is presently believed that administration in a formulation at a high pH (i.e. greater than 8) or at a low pH (i.e. less than 5) is undesirable.

The manner of administering the formulations containing the IL-4 inhibitors of the present invention for systemic delivery can be via subcutaneous, intramuscular, intravenous, oral, intranasal, or vaginal or rectal suppository. Preferably the manner of administration of the formulations containing the IL-4 inhibitor for local delivery is via intraarticular, intratracheal, or instillation or inhalations to the respiratory tract. In addition it may be desirable to administer the IL-4 inhibitor to specified portions of the alimentary canal either by oral administration of the IL-4 inhibitor in an appropriate formulation or device.

For oral administration, the IL-4 inhibitor can be encapsulated. The encapsulated IL-4 inhibitor can be formulated with or without pharmaceutically-acceptable carriers customarily used in the compounding of solid dosage forms. Preferably, the capsule

is designed so that the active portion of the formulation is released at that point in the gastro-intestinal tract when bioavailability is maximized and pre-systemic degradation is minimized. Additional excipients can be included to facilitate absorption of the IL-1 inhibitor. Diluents, flavorings, low melting point waxes, vegetable oils, lubricants, suspending agents, tablet disintegrating agents, and binders can also be employed.

Regardless of the manner of administration, the specific dose is calculated according to the approximate body weight of the patient. Other factors in determining the appropriate dosage can include the disease or condition to be treated or prevented, route of administration and the age, sex and medical condition of the pateint. In certain embodiments, the dosage and administration is designed to create a preselected concentration range of the IL-4 inhibitor in the patient's blood stream. It is believed that the maintenance of circulating concentrations of the IL-4 inhibitor of less than 0.1 ng per ml of plasma may not be effective. It is believed that an appropriate dosage range of the dimeric compounds is between 0.1-1000 ng/ml of plasma. Further refinement of the calculations necessary to determine the appropriate dosage for treatment involving each of the above mentioned formulations is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them without undue experimentation, especially in light of the dosage information and assays disclosed herein. These dosages may be ascertained through use of the established assays for determining dosages utilized in conjunction with appropriate dose-response data.

The IL-4 inhibitor formulations described herein can be used for veterinary as well as human applications and that the term "patient" should not be construed in a limiting manner. In the case of veterinary applications, the dosage ranges should be the same as specified above.

The following examples are intended to illustrate, but not limit, the present invention.

EXAMPLE 1

Expression and purification of IL-4

A. Construction of an IL-4 Expression Plasmid

A plasmid containing the coding sequence for the mature portion of human IL-4 (preceded by methionine) was purchased from R & D Systems (Minneapolis, MN). This sequence was modified using the polymerase chain reaction technique (PCR) to adapt it for cloning into the vector pT5T, which is described in PCT Patent Publication No. WO 91/08285, incorporated herein by reference. The oligonucleotide primers used in the PCR reaction had the sequences:

IL4(5')33 (SEQ.ID.NO.2):

5' CCC **CAT** ATG CAC AAG TGC GAT ATC ACC TTA CAG 3'

IL4(3')37 (SEQ.ID.NO.3):

5' CCC **GGT** ACC TTA TCA GCT CGA ACA CTT TGA ATA TTT C 3'

(overlaps with the IL-4 sequence are underlined; restriction sites added for cloning purposes are boldfaced).

The PCR reaction mixture contained 20 mM Tris-HCl pH 8.2, 10 mM KCl, 6 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 200 μM each of dATP, dCTP, dGTP, and TTP, 20 pmoles of each primer oligo, 1 ng of the template plasmid DNA, and 0 μl (1.25μ) of Pfu polymerase (Stratagene, LaJolla, CA) (total volume = 100 μl). The PCR conditions were 30 cycles of (1 minute at 95°C, 1 minute at 62°C, 1 minute at 72°C), followed by an additional 10 minutes at 72°C.

After the PCR, 80 μl of the reaction mixture was ethanol precipitated and resuspended in 80 μl of 10 mM Tris pH 7.5, 0.1 mM EDTA. 40 μl was digested with NdeI and KpnI and electrophoresed on a 1.2% agarose gel. The DNA band of about 0.4kb was eluted and ligated to plasmid pT5T that had been cut with the same enzymes and gel purified in the same way. The ligation mixture was used to transform Escherichia coli strain HMS174/DE3 (obtained from Dr. F. Willian Studier, Brookhaven National Laboratory, Upton, NY) by the method of Kushner as described in Maniatis

et al., Molecular Cloning: A Laboratory Manual, p.252 (Cold Spring Harbor Laboratory, 1982). Transformants were selected on Luria Broth agar plates containing 50 μg/ml of ampicillin and screened for plasmids containing inserts of the correct size. The IL-4 coding sequence from one such construct was sequenced thoroughly on both strands to verify that it had the expected sequence. It was named pT5T::IL-4. This plasmid was transferred to E. coli strain BL21/DE3 (Dr. F.W. Studier) by transformation. Higher IL-4 expression levels were obtained in E. coli strain BL21/DE3 than in HMS174/DE3. The BL21/DE3 construct was used for further studies.

B. IL-4 expression in E. coli

E. coli BL21/DE3 containing plasmid pT5T::IL-4 were grown to an optical density (O.D.) of 10 and then induced with 150μM isopropylß-D-thiogalactopyranoside (IPTG, Sigma, St. Louis, MO). The cells were harvested by centrifugation when the growth rate slowed to less than 2 OD/hr. The pelleted cells were resuspended in TE (Tris 50mM, pH 8.0, EDTA 5mM) buffer and lysed by three passes through a Gaulin mill (4°C). The IL-4 was recovered as inclusion bodies by differential centrifugation. The inclusion bodies are washed with deionized water to generate washed inclusion bodies (WIBS). About 30-100 grams per 10 liter tank run were recovered.

C. IL-4 Refolding and Purification

WIBS were solubilized in 50mM Tris pH 8.5, 6M guanidine hydrochloride at a ratio of 1:10 (gWIBS:ml buffer) by homogenization using a Brinkmann Polytron (Model PT-3000, Brinkmann Instruments, Inc, Westbury, NY). The mixture was allowed to sit at room temperature for 30 min while stirring. The solubilized WIBS were reduced with 12mM final concentration of dithiothreitol (DTT) (Sigma) and stirred at room temperature for 1hr. The solution was centrifuged (Beckman centrifuge model J2-21, JA-14 rotor, 12,000 RPM) for 25 minutes and the supernatant saved. Oxidized glutathione (Sigma) was added to a 30mM final concentration and the mixture was allowed to sit at room temperature for 10 min while stirring. The refold mixture was

thereafter diluted 10 fold with 50mM Tris pH 10.5, 15.6mM cysteine (Sigma) for a final concentration of 14mM cysteine and left at 4°C for 3-5 days.

D. IL-4 Purification

The refold mix was centrifuged, (JA-10 rotor, 9000 RPM, 35 min) and the supernatant filtered using a Gelman filling machine capsule, 5μm (Gelman Sciences, Ann Arbor, MI). The filtered supernatant was concentrated and diafiltered using 2 Amicon S1Y10 spiral wrap ultrafiltration cartridges in series (Amicon, Beverly, MA). The refold was concentrated approximately 3 fold with a concurrent buffer exchange of 3 fold. The diafiltration buffer was 25mM potassium phosphate pH7.5, 50mM NaCl. Additional buffer was added to adjust concentrated diafiltered pool to approximately 80mM ionic strength.

The concentrated/diafiltered refold mixture was filtered over two 5μM filling machine capsules. The filtered refold was loaded onto a 40ml S-Sepharose column (Pharmacia LKB, Uppsala, Sweden). Column dimensions were 2.6cm x 7.5cm. Buffer A contained 25mM potassium phosphate pH 7.4. The column was previously equilibrated in 5% B (Buffer B is 25mM potassium phosphate pH 7.4, 1M NaCl) before loading and the IL-4 was eluted with a gradient of 5-45% buffer B in 800ml at a flow rate of 10ml/min (0.5%/min). The IL-4 eluted between 150 and 300mM NaCl. Fractions containing IL-4 were pooled based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The pool was concentrated approximately 10 fold using an Amicon stirred cell concentrator with a YM10 membrane (Amicon, Beverly, MA).

The concentrated pool from the S-Sepharose column was split into 2 equal aliquots (approximately 30mg/aliquot by A280) absorbance at 280 nm and each run separately over a 450 ml Sephacryl S-100 (Pharmacia LKB) column (2.6cm x 86cm). The column was previously equilibrated with 25mM KPO4 pH7.4, 120mM NaCl. The column was run at a flow rate of 2ml/min and the IL-4 eluted at molecular weight (mw) equivalent to 14KD based on mw standards. IL-4-containing fractions were pooled

based on SDS-PAGE analysis and concentrated approximately 2-3 fold using an Amicon stirred cell concentrator with a YM10 membrane (Amicon, Beverly, MA).

Final recovery of IL-4 following the typical refold and purification outlined above was between 30-50mg. The TF-1 bioassay results showed that the refolded, purified IL-4 had the same specific activity as commercially available IL-4 (R & D Systems, Minneapolis, MN).

EXAMPLE 2

Construction of IL-4R Clones and Expression

A. Construction of a baculoviral expression clone for IL-4R

The extracellular domain of the human IL-4R was cloned from the human Raji B cell line (ATCC# CCL 86, Rockville, MD). Poly A^+ mRNA was isolated from $9x10^6$ Raji cells using a MicroFastTrack mRNA Isolation kit (Invitrogen, San Diego, CA) according to the manufacturer's directions. cDNA was made from one-third of the mRNA using an Invitrogen cDNA cycle kit (Invitrogen, San Diego, CA). The IL-4R genes in about one-tenth of the cDNA were amplified by PCR using oligonucleotide primers complementary to the 5' and 3' ends of the extracellular domain of the IL-4R. The 5' primer, IL4R(BVp)39, added extra BglII and EcoRI restriction sites to the 5' end of the gene. The 3' primer, IL4R(3'p)51, added extra XbaI, KpnI and BamHI restriction sites at the 3' end of the gene. The sequences of the oligonucleotide primers were:

IL4R(BVp)39 (SEQ.ID.NO.4):

5' CCCAGATCTGAATTCATGGGGTGGCTTTGCTCTGGGCTC 3'

IL4R(3'p)51 (SEQ.ID.NO.5):

5'-CCCTCTAGAGGTACCGGATCCTTATCAGTGCTGCTCGAAGGGCTCCCTGTA-3'

The PCR reaction mixture contained 1X PCR buffer (from a cDNA Cycle kit, Invitrogen, San Diego, CA), 500 uM each of dATP, dCTP, dGTP and TTP, 1 uM of each primer (IL-4R(BVp)39 and IL4R(3'p)51), cDNA, water to 50 ul and 0.6 ul (3 units)

of AMPLITAQ™ (Taq DNA polymerase) (Perkin Elmer, Applied Biosystems Division, Foster City, CA). The PCR reaction conditions were 96°C for 3 minutes, 30 cycles of (94°C, 1 minute; 60°C, 0.5 minutes; 72°C, 2 minutes) and one cycle of 72°C for 10 minutes. Agarose gel analysis of the PCR products revealed a faint band at the expected size of 740 nucleotides. This DNA band was eluted from an agarose gel. One-tenth of the eluted DNA was reamplified by PCR using the same reaction conditions as above. The PCR products were precipitated with ethanol, digested with XbaI and BglII and electrophoresed on an agarose gel. The approximate 740 nucleotide band was eluted and ligated to baculovirus expression plasmid pVL1392 (Invitrogen, San Diego, CA) that had been digested with the same enzymes and purified in the same way. The ligation mixture was used to transform E. coli strain DH5α (catalog #C2022-1, CLONTECH, Inc. Palo Alto, CA). Transformants selected on Luria agar plates containing ampicillin were screened for those containing plasmids of the expected size. The IL-4R genes in 5 such clones were sequenced. None of the clones contained an IL-4R gene with the correct sequence. A plasmid with a correct IL-4R sequence was assembled from two clones that each contained single PCR errors. Clone 3 contained an A to G change at position 359. Clone 14 contained an A to G change at position 163. The IL-4R contains a unique SacI site at position 174, which separates the defective regions of the two clones. The correct 5′ end of clone 3 was joined to the correct 3′ end of clone 14 as follows. Both clones were digested with SacI and SacII, the latter enzyme being unique in the plasmid. The approximate 3330 bp band from clone 3 was gel-purified and ligated to the approximate 7030 bp band from clone 14 that had been gel purified. The ligation mixture was used to transform E. coli strain DH5α and transformants selected on agar plates containing ampicillin. One colony yielded a plasmid with a properly reconstructed IL-4R gene with the correct DNA sequence. This IL-4R plasmid, pVL-IL4R, contains a valine at amino acid 75, which agrees with the sequence reported by Galizzi et al., Int. Immunol. vol. 2, 669-675, 1990).

B. Construction of an E. coli expression clone for IL-4R

The baculovirus expression construct, pVL-IL4R, was modified for expression of the mature amino acid sequence of the extracellular domain of the IL-4R in E. coli using PCR. Codons for the signal sequence were deleted using a primer, IL-4R(5'p)30, that overlaps the IL-4R coding sequence immediately following the signal sequence. The primer adds an NdeI site for cloning purposes. The 3' primer used in the PCR reaction, IL-4R(3'p2)31, contains XbaI, KpnI, and BamHI restriction sites for cloning purposes. The sequences of the primers are:

IL4R(5'p)30 (SEQ.ID.NO.6):

5'-CCCCATATGAAGGTCTTGCAGGAGCCCACC-3'

IL4R(3'p2)31 (SEQ.ID.NO.7):

5'-GGTACCTTCTAGAGGTACCGGATCCTTATCA-3'

The PCR reaction mixture contained 100 pg pVL-IL4R DNA, 2.5 units Pfu polymerase (Stratagene, La Jolla, CA), Pfu polymerase buffer #3 (Stratagene,La Jolla, CA), 0.2 mM dATP, dCTP, dGTP and TTP, and 0.5 uM of each primer (IL4R(5'p)30 and IL4R(3'p2)31. PCR cycling conditions were as described above. The PCR products were ethanol precipitated, digested with KpnI and NdeI and the approximate 660 bp band purified after agarose gel electrophoresis. This DNA fragment was ligated to plasmid pT5T DNA that had been digested with the same enzymes and gel-purified. The ligation mixture was used to transform E. coli DH5α (catalog #C2022-1, CLONTECH Laboratories, Inc., Palo Alto, CA). One transformant yielded a plasmid with a properly constructed IL-4R gene. This plasmid, pT5T-IL4R, was used to transform E. coli strains BL21/DE3 and HMS174/DE3. Expression of the IL-4R as inclusion bodies after IPTG induction in each strain was comparable.

C. Baculoviral Expression of IL-4R

pVL-IL-4R plasmid DNA was co-transfected with BaculoGold DNA (PharMingen, San Diego, CA) into Spodoptera frugiperda (Sf-9) insect cells (Invitrogen, San Diego,

CA) to generate recombinant baculovirus. Primary and secondary viral stocks were prepared. PCR reactions with either viral specific and IL-4R specific primers showed that the virus obtained from the transfections was recombinant virus without detectable contamination by wildtype baculovirus DNA.

An anti-hIL-4R antibody (AB-230-NA, goat polyclonal, R&D Systems, Minneapolis, MN) was used to probe Western blots of culture supernatants to determine optimal sIL-4R expression conditions in Sf-9 cells with respect to multiplicity of infection, time of harvest and serum concentration. Infecting at a multiplicity of infection of 1-2 in Grace's Insect medium (JRH Bioscience, Lenexa, KS) containing 10% serum and harvesting at 3 days post-infection gave the greatest yield of secreted sIL-4R. Higher titer viral stocks were produced and were used for infection of 5 liter Sf-9 insect cell cultures.

EXAMPLE 3

Purification of sIL-4R

A. Preparation of IL-4 Affinity Resin

Affi-Gel 10 resin (Bio-Rad Laboratories, Richmond, CA) was activated by washing with ice cold, 10mM sodium acetate pH 4.5 for 10-15 min using a fitted funnel. The resin was recovered as a moist cake and added to refolded, purified, IL-4 at a concentration of 4-5mg IL-4 / ml of resin. The slurry was allowed to rock for 24-48 hours at 4°C. The unbound sites on the resin were blocked by the addition of 1M ethanolamine (100µl/ml of resin) and rocked at 4°C for 1 hour. Coupling efficiencies were at least 90%. The affinity resin was equilibrated with 50mM Hepes pH 7.3, 150 mM sodium chloride.

B. IL-4R Purification

Approximately 10ml of equilibrated IL-4 affinity resin was added to 5L of IL-4R containing insect cell culture supernatant (previously filtered over .2µm Gelman filling machine capsule), and rotated at 4°C overnight on a roller bottle apparatus (Bellco

Biotechnology, Vineland, NJ). The resin was recovered and subsequently transferred to a column (1.5cm x 5.5cm). The resin was washed with 50mM HEPES, 150mM sodium chloride until the A280 absorbance at 280 nm returned to baseline. The IL-4R was eluted from the column with 150mM sodium acetate pH 3.0, 150mM sodium chloride and neutralized with HEPES pH 9.0. IL-4R containing fractions were pooled based on SDS-PAGE analysis. The pooled fractions were concentrated using Amicon stirred cell concentrator with a YM10 membrane (Amicon, Beverly, MA). IL-4R resolves into several bands at 30-35 kDa in SDS-PAGE due to heterogeneous glycosylation.

Some preparations required an additional size purification step to remove high molecular weight contaminants. In these cases the affinity column pooled concentrate was loaded onto S-100 column (2.6cm x 86cm 450 ml) Sephacryl S-100 (Pharmacia LKB) previously equilibrated with, and run in, 10mM phosphate,200mM sodium chloride at a flow rate of 2ml / min. IL-4R containing fractions were pooled following SDS-PAGE analysis and again concentrated on an Amicon stirred cell. Approximately 3-4mg of purified IL-4R is recovered from 5L of insect cell culture supernatant.

EXAMPLE 4

Construction and Expression of the IL-2Rγ chain (Cys-FLAG construct)

The IL-2Rγ_c cys-FLAG construct was cloned in two steps. The first step entailed cloning a cDNA encoding the extracellular domain of the protein. A cysteine residue was added at the C-terminus of the protein during the cloning process. The second step entailed replacing the 3' end of the cDNA with a new sequence encoding a cysteine followed by two glycines followed by the "FLAG®" epitope (IBI/Eastman Kodak Company, New Haven, CT). FLAG® is an octapeptide having the following amino acid sequence: N-ASP-Try-Lys-Asp-Asp-Asp-Asp-Lys-C (SEQ.ID.NO.1). The FLAG epitope was added to facilitate purification of the protein by utilizing the monoclonal antibody to FLAG, referred to as ANTI-FLAG M2 (IBI, Kodak), linked to resin for affinity purification.

A. Cloning of the IL-2R-gamma extracellular domain and adding a C-terminal cysteine.

The extracellular portion of the IL-2Rγ_c gene was cloned from the human Raji B cell line (ATCC # CCL 86). mRNA was isolated from 3 x 10^6 Raji cells using a Micro-FastTrack mRNA Isolation Kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. cDNA copies of one tenth of the mRNA were made using a cDNA Cycle Kit (Invitrogen, San Diego, CA). The IL-2Rγ_c genes in one fifth of the Raji cDNA were then amplified by PCR using oligonucleotide primers complementary to the 5' and 3' ends of the extracellular portion of the IL-2Rγ_c sequence. The 5' oligonucleotide primer, IL2RG(5'p)38, added an XbaI restriction site to the 5' end of the gene. The 3' oligonucleotide primer, IL2RG(3'p)54, added a cysteine codon (cys 255) to the 3' end of the extracellular portion of the gene, changed the codon for Serine 251 from TCA to TCG to create a BstBI restriction site, and added a BamHI restriction site to the 3' end. The oligonucleotide primers used were:

IL2RG(5'p)38 (SEQ.ID.NO.8):

5' CCCTCTAGATGTTGAAGCCATCATTACCATTCACATCC 3'

IL2RG(3'p)54 (SEQ.ID.NO.9):

5'CCCGGATCCTCATTAGCAATTCTCTTTCGAAGTATTGCTCCCCCAGTGGATTGG 3'

(overlaps with IL-2Rγ_c sequence are underlined)

The PCR reaction mixture contained 1x "PCR Buffer" (from a cDNA Cycle Kit, Invitrogen, San Diego, CA), 500μM each of dATP, dCTP, dGTP, TTP, 20pmole of each primer [IL2RG(5'p)38 and IL2RG(3'p)54], Raji cell cDNA, water to 50μl and 0.5μl (2.5units) of AMPLITAQ (Taq DNA Polymerase). The PCR reaction conditions were 30 cycles of: 96°C, 3 minutes; 95°C, 1 minute, 60°C, 1 minute, 72°C, 1:30 minutes, followed by a 10 minute cycle at 72°C. After the PCR reaction was completed, 40 μl of the reaction mixture was diluted to 80 μl with water then passed over a spin column (ChromaSpin-100, ClonTech, Palo Alto, CA). 20 μl was digested with XbaI and HindIII

and electrophoresed on a 0.8% agarose gel. The band of about 0.8kb was eluted and ligated to baculovirus expression plasmid pVL1392 (Invitrogen, San Diego,CA) that had been cut with the same enzymes and gel purified in the same way. The ligation mixture was used to transform E. coli strain DH5α. Colonies selected on Luria Broth agar plates containing 50 μg/ml of ampicillin were screened for plasmids containing inserts of the correct size and the inserted gene from one such construct was sequenced thoroughly on both strands to verify that it had the expected sequence. This clone was named 81-4-2.

B. Construction of a baculovirus expression clone for IL-2Rγ_c with a "FLAG" epitope at the 3' end.

Clone 81-4-2 was altered to add the following sequence: Gly Gly Asp Tyr Lys Asp Asp Asp Asp Lys Stop Stop (SEQ.ID.NO.10) at the carboxy terminus of the IL-2R-gamma by PCR mutagenesis using a 3' oligonucleotide primer [IL2RG(FLAG)67] that contained the changes and overlapped the 81-4-2 sequence and a 5' oligonucleotide primer [IL2RG(494)27] that overlapped an internal sequence of 81-4-2; the template was 81-4-2 plasmid DNA. The underlined region of the sequence encodes the "FLAG" epitope (IBI/Eastman Kodak Company, New Haven, CT). After the PCR the amplified DNA fragment was purified by running over a spin column, as described above, and cutting with BamHI and Bpu1102I (the latter enzyme cuts near the 3' end of the IL2 receptor gamma gene) and eluting the 101 bp DNA fragment from an agarose gel. The modified fragment was ligated to plasmid 81-4-2 that had been cut with the same two restriction enzymes and gel purified in the same way. The result was to substitute the FLAG modified sequence for the previous end of the gene in clone 81-4-2. A clone that was sequenced across the Bpu1102I to BamHI region on both strands and found to be correct was selected for further use and was named 90-1-5. This plasmid was then used to transform E. coli DH5α. The sequences of the oligonucleotide primers used were:

IL2RG(494)27 (SEQ.ID.NO.11):

5' CAAACTGAGTGAATCCCAGCTAGAACT 3'

IL2RG(FLAG)67 (SEQ.ID.NO.12):

5'-CCCGGATCCTCATTACTTATCGTCATCGTCTTTGTAG

TCGCCTCCGCAATTCTCTTTCGAAGTATTG 3'

(overlaps with the IL-2rγ_c sequence in plasmid 81-4-2 are underlined)

The PCR conditions used were 10mM Tris pH 8.3, 50mM KCl, 1.5mM $MgCl_2$, 0.001% gelatin (Sigma Chemical Company, St. Louis, MO; catalogue # G2500), 200μM each of dATP, dCTP, dGTP, TTP 20pmole of each primer [Il2RG(494)27 and Il2RG(FLAG)67], ~1ng of template DNA (plasmid 81-4-2), water to 100μl, and 0.5μl (2.5units) of AMPLITAQ (Taq DNA Polymerase). The PCR mixture was cycled 30 times: 96°C, 3 minutes; 95°C, 1 minute, 55°C, 1 minute, 72°C, 1 minute, followed by 10 minutes at 72°C.

C. Construction of an E. coli expression clone for IL-2Rγ_c with a carboxy terminal cysteine.

The baculovirus expression construct, 81-4-2, was modified to express the mature amino acid sequence of the extracellular portion of IL-2Rγ_c in E. coli using PCR. This clone retained the cysteine codon (cys 234) at the 3' end of the gene. Codons for the first 22 amino acids were deleted using a primer [IL2RG(72p)33] that overlapped the IL-2Rγ_c sequence beginning at codon 23 and added an NdeI site, containing the initial methionine codon, to the 5' end. After the PCR the DNA was cleaned up by running it over a spin column, cutting with NdeI and BamHI, and eluting the 707 bp fragment from an agarose gel (as described previously). The eluted fragment was ligated to plasmid pT5T DNA cut with the same enzymes and eluted from a gel in the same way. After transforming E. coli strain DH5α with the ligation mixtures one of the resulting plasmids was sequenced on both strands and found to have the expected sequence. This clone, called 91-1-1, was used to transform E. coli

host strain BL21/DE3 for expression studies. This strain with clone 91-1-1 was cultured and upon induction with IPTG was shown to express IL-2Rγ at a high level. The oligonucleotide primers used were:

IL2RG(72p)33 (SEQ.ID.NO.13):

5' CCCCATATGCTGAACACGACAATTCTGACGCCC 3'

IL2RG(3'p)54 (SEQ.ID.NO.14):

5' CCCGGATCCTCATTAGCAATTCTCTTTCGAAGTAT TGCTCCCCCAGTGGATTGG 3'

(overlaps with 81-4-2 sequence are underlined)

The PCR reaction mixture contained 20mM Tris pH 8.75, 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgCl_2$, 0.1% Triton X-100, 0.1 mg/ml bovine serum albumin, 200μM each of dATP, dCTP, dGTP, TTP, 20pmole of each primer [IL2RG(72p)33 and IL2RG(3'p)54], ~1ng of template DNA (plasmid 81-4-2), water to 100μl and 1μl (2.5units) of Pfu DNA Polymerase (Stratagene, San Diego, CA). The PCR mixture was cycled 30 times: 96°C, 3 minutes; 95°C, 1 minute; 65°C, 1 minute; 72°C, 2 minutes, followed by a 10 minute cycle at 72°C.

D. Construction of an E. coli expression clone for IL-2Rγ without a carboxy terminal cysteine.

Plasmid clone 91-1-1 was modified to make a clone coding for the mature, extracellular, portion of IL-2rγ that did not contain a carboxy terminal cysteine. An oligonucleotide, IL2RG(3'wt)41, was designed that overlapped the 3' end of the coding sequencebut deleted the terminal cysteine codon. This oligo was used with the 5' oligo il2rg(72p)33 described above and plasmid 91-1-1 as template in a polymerase chain reaction. The sequences of the oligonucleotide primers used were:

IL2RG(72p)33 (SEQ.ID.NO.15):

5' CCCCATATGCTGAACACGACAATTCTGACGCCC 3'

IL2RG(3'wt)41 (SEQ.ID.NO.16):

5' CCCGGATCCTCATTAATTCTCTTTCGAAGTATTGCTCCCCC 3'

(overlaps with 91-1-1 sequence are underlined)

The PCR reaction mixture contained 20mM Tris pH 8.75, 10mM KCl, 10mM $(NH_4)_2SO_4$, 2mM $MgCl_2$, 0.1% Triton X-100, 0.1 mg/ml bovine serum albumin, 200μM each of dATP, dCTP, dGTP, TTP, 20pmole of each primer [Il2RG(72p)33 and Il2RG(3'wt)41], ~1ng of template DNA (plasmid 91-1-1), water to 100μl and 1μl (2.5u) of Pfu DNA Polymerase (Stratagene, San Diego, CA). The PCR reaction conditions were 96°C for 3 minutes, 30 cycles of (95°C, 1 minute; 65°C, 1 minute; 72°C, 2 minutes), followed by 72°C for 10 minutes.

After the PCR the resulting gene fragment was digested with NdeI and BamHI, gel purified, and ligated to pT5T vector DNA that had been cut with the same enzymes. The ligation mixture was used to transform E. coli strain DH5 alpha as described above. The plasmid in one of the resulting transformants was sequenced on both strands and found to have the correct sequence. This clone was named 96-1-1 and transformed into E. coli host strain BL21/DE3 for expression studies. E. coli BL21/DE3 pT5T::IL-2Rγ_c (96-1-1) was cultured and induced with IPTG at lab bench scale and shown to express IL-2Rγ at a high level.

E. Expression of sIL-2Rγ_c in Insect Cell Culture

Plasmid DNA was prepared from E. coli DH5α containing pVL1392::IL-2Rγ_c-FLAG (90-1-5) utilizing a commercial plasmid purification kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions.

This plasmid DNA (5μg), insect viral DNA (0.5μg BaculoGold DNA, #21100D, PharMingen, San Diego, CA) and cationic liposome solution (30μl, Lipofectin, #18292-011, Gibco BRL, Gaithersburg, MD) were mixed with 3 ml of Grace's medium (JRH Bioscience, Lenexa, KS) and then used to transfect Sf9 insect cells (PharMingen, San

Diego, CA). A high titer viral stock was prepared by standard baculoviral culture methods (Summers, M.D., and Smith, G.E. (1987) *A Manual of Methods of Baculovirus Vectors and Insect Cell Culture Procedures*, Texas Agriculture Experiment Station Bulletin No. 1555). The kinetics of secretion of IL-2Rγ_c were followed by Western blot utilizing anti-FLAG monoclonal antiserum, M2 (IBI/Kodak, New Haven, CT) and an appropriately tagged secondary antiserum; the optimal harvest time determined to be 56-64 hours post-infection.

EXAMPLE 5

Purification of sIL-2Rγ_c (cys-FLAG construct)

Soluble IL-2Rγ_c was purified from insect cell culture supernatants by affinity chromatography using an ANTI-FLAG M2-antibody affinity resin. Anti-FLAG M2 Antibody resin appropriate secondary antibody (IBI/Eastman Kodak Company, New Haven, CT.) was washed extensively and equilibrated with 50mM HEPES pH 7.3, 150mM sodium chloride. Approximately 25ml of the equilibrated anti-FLAG affinity resin was added to 5L of IL-2Rγ_c containing insect cell culture supernatant (.2μ filtered) and rotated at 4°C on a roller bottle apparatus. The resin was recovered, transferred to a column (2.5cm x 5cm) and washed with phosphate buffered saline.

The IL-2Rγ_c-FLAG was eluted from the column with 100mM glycine pH 3.0 and neutralized with HEPES pH 9.0. The IL-2Rγ_c containing fractions were pooled based on SDS-PAGE analysis and run over Sephacryl S-100 (Pharmacia LKB) sizing column (2.6cm x 86cm) to remove high molecular weight contaminants and IL-2Rγ_c-FLAG aggregates. The column was equilibrated with, and run in 10mM phosphate, 200mM sodium chloride at a flow rate of 2ml / min. IL-2Rγ_c containing fractions were pooled based on SDS-PAGE analysis and concentrated on an Amicon stirred cell. Approximately 1-2mg of purified IL-2Rγ_c was recovered from 5L of insect cell culture supernatant. IL-2Rγ-FLAG resolves into multiple bands at 40-42 kDa in SDS-PAGE due to heterogeneous glycosylation.

EXAMPLE 6

Preparation of the Heterodimer and Homodimer

A. Conditions for PEGylation of IL-2Rγ_cFLAG

The purified IL-2Rγcys-FLAG subunit was reduced with varying concentrations of dithiothreitol (DTT) to determine the reduction concentration which facilitates addition of only one PEG group to the protein. The protein at 16 to 90 μM was reduced with 5 fold excess DTT and was then dialyzed. The IL-2Rγ_c FLAG was PEGylated efficiently with 3-4 fold excess 8K methoxy-PEG-maleimide.

B. Mono-PEGylation of IL-4R

Affinity purified IL-4R (104 μM) was reduced with an 8 fold molar excess of dithiothreitol (DTT, Sigma) at 832μM. Incubate for 30 min at room temperature. The mixture was dialyzed against 10mM phosphate pH 7.5, 200mM sodium chloride for 4hr at 4°C using Spectra/Por #1 dialysis membranes with a molecular weight cut off of 6000-8000 da (Spectrum Medical Industries, Houston TX). Approximately 80% or 88μM of the reduced IL-4R is recovered following dialysis. The reduced IL-4R was PEGylated with 8K methoxy-PEG-Maleimide (Synergen Lot #1871-53) at a 4:1 PEG:protein ratio, 352μM:88μM. React for 1-2 hr at room temperature. PEGylation efficiency was approximately 70-80%. The mono-PEGylated IL-4R was purified by gel filtration (Superose 12 10/30 column, Pharmacia LKB) run in 10mM phosphate pH7.5, 200mM sodium chloride at a flow rate of 0.3ml/min. The mono-PEGylated-IL-4R containing fractions were pooled following SDS-PAGE analysis, and concentrated on an Amicon stirred cell with a YM10 membrane (Amicon, Beverly, MA). Protein concentration was determined by Bradford protein assay.

C. Preparation of Polyethylene glycol (PEG) cross-linked sIL-4:sIL-2Rγ_c

The sIL-4R and sIL-2Rγ_c proteins were joined together using bi-functional polyethylene glycol (PEG) reagents. The following three different PEG reagents for cross-linking the proteins were used: PEG 8000 *bis*-maleimide, PEG 20,000 *bis*-

maleimide, and PEG 3400 *bis*-maleimide. These cross-linking reagents were prepared according to the procedures described in U.S. Application Serial No. 08/259,413, filed June 14, 1994, incorporated herein by reference.

Affinity purified IL-2Rγ_c (83μM, Tris pH 7.0 50mM) was reduced with dithiothreitol (DTT, 5fold excess at 416μM) 30 minutes at room temperature. DTT was removed by dialysis against Tris 50mM pH 7.0, 4 hr, 4°C.

Affinity purified IL-4R (80μM in Tris pH 7.0 50mM) was reduced with 8 fold excess DTT at 640μM and incubated 30 min, at room temperature. The reduced IL04R was run over size exclusion column to remove DTT (Bio-spin 6, Biorad #732-6002). About 80% or 64μM of the reduced receptor was recovered. The purified IL-4R was PEGylated with PEG 3400 bis-maleimide (Synergen Lot #2102-114) at a 4:1 excess PEG:protein, 256μM:64μm and allowed to react 1 hr at room temperature. PEGylation efficiency was 50-80%. Mono-PEGylated receptor was purified utilizing gel filtration (Superose 12 HR 10/30, Pharmacia #17-0538-01 phosphate buffered saline, 200mM, 0.3 ml/min). Mono-PEG IL-4R was pooled and immediately concentrated by microcentrator (Microcon, Amicon, Beverly, MA) and the protein concentration was determined by the Bradford protein assay.

The maleimide-PEG 3.K-IL-4R was added to 1.5 to 4 fold excess reduced IL-2Rγ_c to facilitate heterodimer formation. The mixture was allowed to react for 1 hr at room temperature and then overnight at 4°C. At least 50% of the mono-PEG IL-4R was converted to heterodimer.

The IL-4R/IL-2Rγ_c heterodimer was purified from other proteins by gel filtration (Superose 12 HR 10/30 or Superdex 75 HR 10/30, Pharmacia #17-1047-01, same buffer as above). Run pooled peak fractions were run on reducing SDS-PAGE to assess purity and the protein concentration was determined by Bradford. Bioactivity of the heterodimer was assessed in the TF-1 bioassay described in Example 7.

Purified 3.4K PEG heterodimer run on reducing SDS-PAGE is contaminated with IL-2Rγ_c. The heterodimer is ~75% of the total protein. The contaminating IL-2Rγ_c appears to be aggregating with the heterodimer and, thus, is migrating with this high molecular weight molecule on the gel filtration column. A second purification step can

be used to obtain a more pure product. To facilitate further purification of dimeric constructs, two approaches will be taken. Firstly, salt, detergent and mild reducing agent will be tried to disassociate the aggregates of IL-2Rγ and, thus, allow better resolution in the current method. Secondly, other chromatographic methods will be attempted which distinguish the more hydrophilic PEGylated proteins from the unmodified proteins, such methods include ion exchange, hydrophobic interaction, and reverse phase chromatography.

The same procedure was used to prepare the PEG_{20000} and PEG_{8000} heterodimers. Purified 20K and 8K PEG heterodimer preparations were judged by SDS-PAGE to be 70-90% pure.

D. Preparation of sIL-4R-Homodimer PEG_{20K}

The purified IL-4R was reduced with DTT (600μM) and recovered from spun size exclusion column as decribed above. The IL-4R was reacted with PEG_{20K} *bis*-maleimide at 2:1 excess protein to PEG at 4°, overnight. The mixture was purified by gel filtration (as above). The purified homodimer-PEG_{20K} used for the bioassay appears to be 90% pure by reducing SDS-PAGE.

EXAMPLE 7

In vitro Assays

A. Culture of TF-1 Cells

TF-1 cells (ATCC # CRL-2003) are human immature erythroleukemic cells which are stimulated to proliferate by IL-5, GM-CSF, EPO and IL-3. IL-4 and IL-6 extend the survival of TF-1 cells according to Kitamura et al., J. Cellular Physiology, 140:323-334 (1989). The cell line was passed every 2-3 days RPMI-1640 (Bio-Whittaker #12-115V) plus Pen-Strep at 100 units/ml, 100 mcg/ml, (Irvine Scientific, Walkersville, MD, Santa Ana, CA #9366) plus 10% fetal bovine serum defined, (HyClone Laboratories, Inc., Logan, UT #A-1111-L) with IL-5, 5 ng/ml recombinant Sf21, human sequence (R&D Systems, Minneapolis, MN) 37°C, 5% CO_2, tissue culture incubator.

B. Preparation of TF-1 cells for bioassay

TF-1 cells at 0.8 to 1.2 x 10^6 cells/ml were harvested by centrifugation (Beckmann GP Centrifuge, 900 rpm, 5 min) and washed twice in complete RPMI-medium (described above). The cells were returned to the incubator for 2-4 hours and then washed twice more, and titered. Cells were added to final concentration of 2-3 x 10^5/ml in the microwells.

C. TF-1 Bioassays

1. Titration of IL-4 in vitro Activity in TF-1 Proliferation Assay

IL-4 was diluted into microtiter wells (Corning Cell Wells, #25860, 96-well, flat bottom). The IL-4 concentration range tested was 0.05 pM to 3000 pM IL-4 (recombinant (E. coli), human, carrier-free, R&D Systems, Minneapolis, MN) was used as the standard. Cells were added at 2-3x10^4/100μl/well. A medium blank and a cell control were set up in triplicate as well as all the experimental samples.

The wells were incubated at 37°, CO_2 tissue culture incubator for 66 to 68 hrs. 3-[4,5 Dimethyl thiazol-2-yl]-2,5 diphenyl tetrazolium bromide, (MTT, Sigma) (10μl/well of 5 mg/ml in PBS, 0.2 μ filter sterilized) was added and mixed gently while protect from light. The wells were incubated 37° for 6 hours and thereafter solubilized with DMF-SDS (N,N-dimethyl foramide 50%, sodium dodecyl sulfate 20% to pH 4.7 with acetic or hydrochloric acid and filtered (0.2μ), 50μl/well. The wells were then incubated overnight at 37°C, read at 570-660 on VMAX Kinetic Microplate Reader, (Molecular Devices Corp., Palo Alto, CA) and analyzed with SOFTMax software from Molecular Devices.

Table 1 shows data for the proliferative effect of measuring IL-4 concentrations on TF-1 cells as measured in this MTT uptake assay. The observed proliferative response is TF-1 cells without IL-4 (Abs = .19) to maximal stimulation of TF-1 cells at Abs of 0.84. The EC_{50}'s (effective concentration which gives half maximal response) for these two titrations of the standard IL-4 are 4pM and 5.1pM. The range of EC_{50}'s was 1-10pM.

Table 1 Effect of increasing concentrations of IL-4 on TF-1 cell proliferation. Two titrations of IL-4 standard

IL-4, nM	Absorbance 570-660			
5.00E-05	0.202	±0.005	0.184	±0.012
0.0001	0.192	±0.014	0.186	±0.002
0.0002	0.21	±0.008	0.186	±0.004
0.0004	0.215	±0.014	0.202	±0.013
0.00081	0.26	±0.004	0.213	±0.006
0.0016	0.349	±0.032	0.28	±0.005
0.0032	0.473	±0.022	0.382	±0.025
0.0065	0.592	±0.017	0.556	±0.006
0.013	0.707	±0.004	0.7	±0.017
0.026	0.772	±0.029	0.799	±0.033
0.052	0.773	±0.004	0.825	±0.032
0.103	0.797	±0.013	0.827	±0.05
0.207	0.849	±0.012	0.846	±0.058
0.414	0.816	±0.032	0.838	±0.04
0.828	0.842	±0.018	0.886	±0.055
1.66	0.818	±0.019	0.886	±0.046
3.31	0.843	±0.016	0.867	±0.058

2. Titration of IL-4 Receptor Constructs as Inhibitors of IL-4 dependent TF-1 Proliferation Assay

A constant concentration of IL-4 (2-20 pM) (best results with 6-10pM) was added to all the test wells. The sIL-4R constructs were diluted into the IL-4, incubated at 37°, 30 min and then washed TF-1 cells were added to a final concentration of 2-3x10^4 cells/100μl/well. The microtiter plates were incubated, processed, and read as described above.

The MonoPEGylated sIL-4R-PEG8K was compared to sIL-4R in the inhibition of TF-1 proliferation assay (Table 2). The concentration of the constant IL-4 in this assay is 20pM. The monoPEGylated IL-4R had slightly less (10-15%) bioactivity than the soluble IL-4R. The difference is not significant and is within experimental variation of the protein concentration determination and the bioasay.

Table 2 Effects of increasing concentrations of sIL-4R and sIL-4R-mono-PEG 8K on inhibition of IL-4 stimulated proliferation of TF-1 cells

IL-4R, nM	Absorbance, 570-660			
	sIL-4R		sIL-4R-mono PEG 8K	
10.50	0.154	±0.005	0.2	±0.01
5.25	0.233	±0.006	0.308	±0.016
2.6	0.344	±0.005	0.38	±0.008
1.3	0.425	±0.016	0.458	±0.009
0.66	0.47	±0.015	0.477	±0.003
0.33	0.499	±0.019	0.498	±0.01
0.16	0.531	±0.011	0.524	±0.018
0.08	0.529	±0.031	0.531	±0.026
0.04	0.553	±0.023	0.582	±0.031

3. Comparisons of sIL-4R, mono-pegylated sIL-4R, homodimer and heterodimer.

The PEG_{20K} IL-4R constructs were compared in the TF-1 assay with a constant concentration of IL-4 at 20pm (Table 3). The monopegylated IL-4R-PEG_{20K} has the same bioactivity as the unmodified sIL-4R indicating that modification of that cysteine with PEG has no detectable effect on the in vitro activity of the IL-4R.

The IL-4R homodimer and the heterodimer PEG20K both have slightly increased bioactivity (2-3 fold) as compared to sIL-4R. The IC_{50}'s (inhibitory concentration which inhibits 50% of the proliferative activity of IL-4) reflects the affinity of the molecule for IL-4. The IL-4R homodimer has an IC_{50} which is less than would be expected for two IL-4R subunits acting independently. Thus, both dimers have some potential efficacy as IL-4 inhibitors.

Table 3 Effects of increasing concentrations of sIL-4R, IL-4R-mono-PEG 20K, IL-4R-Homodimer-PEG 20K and Heterodimer-PEG 20K on IL-4 stimulated proliferation of TF-1 cells

IL-4 Receptor constructs, nM	Absorbance 570-660						Heterodimer, nM	Absorbance 570-660	
	sIL-4Rwt		IL-4R-mono-20K		Homodimer-20K			Heterodimer-20K	
0.023	---	---	---	---	0.38	±0.04	0.017	0.373	±0.003
0.046	0.406	±0.013	0.363	±0.013	0.365	±0.027	0.035	0.394	±0.02
0.081	0.406	±0.019	0.363	±0.008	0.349	±0.017	0.071	0.381	±0.014
0.162	0.39	±0.01	0.362	±0.004	0.336	±0.008	0.143	0.381	±0.025
0.325	0.405	±0.025	0.361	±0.006	0.284	±0.021	0.287	0.352	±0.027
0.65	0.354	±0.023	0.346	±0.004	0.215	±0.015	0.575	0.272	±0.005
1.3	0.285	±0.008	0.288	±0.002	0.147	±0.004	1.15	0.202	±0.015
2.6	0.205	±0.017	0.217	±0.005	0.088	±0.003	2.3	0.122	±0.002
5.25	0.121	±0.003	0.136	±0.01	0.06	±0.002	4.6	0.079	±0.003
10.5	0.076	±0.001	0.072	±0.004	---	---	--	---	---

4. Comparison of sIL-4R with heterodimer-PEG_{8K}

Heterodimer-PEG_{8K} has increased bioactivity in the in vitro assay as shown in Table 4. The constant concentratio of IL-4 was 10pm, which is within the linear range of the IL-4 response. The IC_{50} for the heterodimer-PEG_{8K} is 3.4 lower than the IC_{50} for sIL-4R. The heterodimer-PEG_{8K} has greater bioactivity than the heterodimer-PEG_{20}.

Table 4 Effects of increasing concentrations of sIL-4R and Heterodimer-PEG-8K on inhibition of IL-4 stimulated proliferation of TF-1 cells

IL-4R, nM	Absorbance, sIL-4R		Het 8K, nM	Absorbance, Heterodimer-8K	
0.04	0.965	±0.015	---	---	---
0.08	0.857	±0.028	0.0078	1.013	±0.056
0.16	0.795	±0.044	0.0156	0.946	±0.027
0.33	0.687	±0.018	0.0313	0.92	±0.08
0.66	0.543	±0.039	0.0625	0.788	±0.037
1.3	0.395	±0.021	0.125	0.623	±0.029
2.6	0.304	±0.014	0.25	0.445	±0.033
5.25	0.232	±0.008	0.5	0.301	±0.024
10.5	0.213	±0.01	1	0.241	±0.026

5. Comparison of sIL-4R with heterodimer-$PEG_{3.4K}$.

The in vitro data for heterodimer-$PEG_{3.4K}$ are presented in Table 5. Two independent preparations of the heterodimer-$PEG_{3.4K}$ were constructed, purified and assessed with consistent results. The heterodimer-$PEG_{3.4K}$ has bioactivity about 38-fold better than sIL-4R in this assay. It was 18- and 22-fold for the two lots in a previous assay. These data indicate that IL-2Rγ_c significantly stabilizes the IL-4:IL-4R complex.

Table 5 Effects of increasing concentrations of sIL-4R, and Heterodimer 3.4K (two lots) on IL-4 stimulated proliferation of TF-1 cells

IL-4Receptor constructs		Absorbance 570-660 center					
sIL-4R, nM	Het 3.4K nM	sIL-4R		Heterodimer 3.4K Lot 1		Heterodimer 3.4K Lot 2	
0.00125	0.0001	0.542	±0.015	0.564	±0.016	----	---
0.0025	0.0002	0.546	±0.028	0.546	±0.006	0.57	±0.023
0.005	0.00039	0.54	±0	0.547	±0.008	0.588	±0.037
0.01	0.00078	0.547	±0.028	0.528	±0.019	0.54	±0.01
0.02	0.00156	0.555	±0.024	0.504	±0.014	0.515	±0.009
0.04	0.00312	0.526	±0.038	0.466	±0.012	0.44	±0.009
0.082	0.00625	0.473	±0.015	0.404	±0.014	0.339	±0.005
0.164	0.0125	0.43	±0.007	0.287	±0.032	0.237	±0.02
0.33	0.025	0.339	±0.005	0.239	±0.01	0.22	±0.01
0.66	0.05	0.266	±0.011	0.192	±0.02	0.193	±0.003
1.31	0.1	0.217	±0.005	0.189	±0.009	0.183	±0.008
2.62	0.2	0.2	±0.012	0.177	±0.016	0.189	±0.009
5.25	.042	0.19	±0.017	0.178	±0.011	0.184	±0.001
10.5	.867	0.185	±0.01	---	---	0.18	±0.005

6. Comparison of IC_{50}s.

The in vitro bioactivities of the IL-4R-PEG constructs and the unmodified sIL-2Rγ_c in the TF-1 assays are compared to the bioactivity of sIL-4R in Table 6. The IC_{50} for sIL-4R in a particular assay is divided by the IC_{50} for that protein construct to give a relative in vitro activity for each. The mono-pegylated IL-4R's are similar to unmodified sIL-4R, indicating that the addition of PEG does not reduce the IL-4 inhibitory activity of the molecules. IL-2Rγ_c has virtually no activity in this inhibitory assay. The homodimeric and heterodimeric receptor constructs have improved bioactivity as IL-4 inhibitors. The heterodimeric IL-4R:IL-2Rγ_c constructs have further improved inhibitory activity as the PEG cross-linker size is reduced. These data indicate that the IL-2Rγ_c increases IL-4 inhibitory activity and provides better IL-4 inhibitors as the cross-linker is reduced in length.

Table 6 Comparisons of IC_{50}'s for each IL-4R construct based on its inhibition of IL-4 stimulated TF-1 cell proliferation

IL-4 Receptor Construct	Relative IC_{50}[a]
sIL-4R	1.0
sIL-2Rγ	0.006
sIL-4R-PEG 8K	0.9
sIL-4R-PEG 20K	0.9--1.0
IL-4R-Homodimer-PEG 20K	3.3[b]
Heterodimer-PEG 20K	1.3--2.3
Heterodimer-PEG 8K	3.4--4.4
Heterodimer-PEG 3.4K	18--38

[a] IC_{50} of sIL-4R/IC_{50} of receptor construct, Data are presented in Tables 2 through 5.

[b] Homodimer has two IL-4R subunits per molecules; thus 3.3/2 is 1.6 for equivalence in this comparison.

EXAMPLE 8

Dissociation Rates of the IL-4R Constructs

The dissociation rates of the IL-4R constructs from IL-4 were determined. IL-4 was immobilized on a BIAcore™ chip and analyzed on a BIAcore™ System (Pharmacia Biosensor AB, Uppsala, Sweden) according to the manufacturer's instructions. Table 7 reports the results of the analysis.

Table 7 Dissociation Rates of IL-4 Receptor constructs from immobilized IL-4 before and after IL-4 wash (1μM)

Receptor Construct	Immobilized to chip at low density	$t_{1/2}$ (min)	
		Buffer Flow	Buffer (After IL-4 Wash)
sIL-4R	IL-4	0.8--1.6 min	zip
Homo 20K	IL-4	8--16 min	zip
Het 20K	IL-4	10 min	67 min
Het 8K	IL-4	14 min	104 min
Het 3.4K	IL-4	13.4 min	124 min
	372 Resonance Units IL-4 immobilized to chip, IL-4 WT		

zip = all bound resonance units are washed off

The foregoing description of the invention is exemplary for purposes of illustration and explanation. It will be apparent to those skilled in the art that changes and modifications are possible without departing from the spirit and scope of the invention. It is intended that the following claims be interpreted to embrace all such changes and modifications.

What is claimed is:

1. A dimeric compound having affinity for IL-4 comprising R_1-X-R_2, wherein:

R_1 is an IL-4 receptor (IL-4R);

R_2 is an IL-4R or an IL-2 receptor gamma chain (IL-2Rγ_c); and

X is a polymeric spacer.

2. The dimeric compound of claim 1, wherein R_2 is the IL-4R.

3. The dimeric compound of claim 1, wherein R_2 is the IL-2Rγ_c.

4. The dimeric compound of claim 1, wherein X is a non-peptidic, polymeric spacer.

5. The dimeric compound of claim 4, wherein X is polyethylene glycol.

6. The dimeric compound of claim 5, wherein the polyethylene glycol has a molecular weight in the range of about 1kD to about 20kD.

7. The dimeric compound of claim 6, wherein the polyethylene glycol has a molecular weight of up to about 8kD.

8. The dimeric compound of claim 6, wherein the polyethylene glycol has a molecular weight of up to about 3.4kD.

9. The dimeric compound of claim 1, wherein R_1 is IL-4R, R_2 is IL-2Rγ_c, and X is polyethylene glycol.

10. A method for preparing a heterodimeric compound having affinity for IL-4 comprising:

(a) obtaining an IL-4 receptor and a IL-2Rγ_c;

(b) reacting said IL-4 receptor with a polymeric spacer to form an IL-4 receptor/polymer complex; and

(c) reacting said IL-2Rγ_c to the IL-4 receptor/polymer complex to form a heterodimeric compound.

11. The method of claim 10, wherein said IL-4 receptor and IL-2γ_c are recombinantly produced.

12. The method of claim 10, furthering comprising the step of:

(d) purifying said heterodimeric compound.

13. A method for treating an IL-4 mediated disease comprising administering to a patient in need thereof a therapeutically effective amount of the dimeric compound of claim 1.

14. The method of claim 13, wherein the IL-4 mediated disease is mediated by a deleterious increase in IgE production.

15. The method of claim 13, wherein the IL-4 mediated disease is allergic rhinitis, asthma, atopic dermatitis, eczema.

16. The method of claim 13, wherein the IL-4 mediated disease is infections caused by yeast, an intracellular parasite or a virus.

17. The method of claim 16, wherein the virus is HIV.

18. A pharmaceutical composition comprising the dimeric compound of claim 1 in a pharmaceutically-acceptable carrier.

19. A method of detecting or quantifying IL-4 in a sample, comprising:

(a) contacting the dimeric compound of claim 1 with the sample;

(b) allowing said dimeric compound to bind to IL-4; and

(c) detecting or quantifying IL-4 bound to said dimeric compound.

20. A method of purifying IL-4 in a sample, comprising:

(a) contacting the dimeric compound of claim 1 with the sample;

(b) allowing said dimeric compound to bind to IL-4;

(c) dissociating the IL-4 from the dimeric compound; and

(d) collecting the dissociated IL-4.

INTERNATIONAL SEARCH REPORT

Int. ional Application No
PCT/US 95/13101

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/715 A61K38/20 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCIENCE, vol. 262, 17 December 1993 LANCASTER, PA US, pages 1874-1877, M. KONDO ET AL. 'Sharing of the Interleukin-2 (IL-2) Receptor gamma Chain Between Receptors for IL-2 and IL-4' cited in the application see page 1876, left column, paragraph 3 - right column, paragraph 1 --- -/--	1,4-8, 10,13, 18,19

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 January 1996	26. 01. 96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Fuhr, C

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCIENCE, vol. 262, no. 5141, 17 December 1993 LANCASTER, PA US, pages 1880-1883, S.M. RUSSEL ET AL. 'Interleukin-2 Receptor gamma Chain: A Functional Component of the Interleukin-4 Receptor' cited in the application see page 1882, middle column, paragraph 2 - right column, paragraph 3	1,4-8, 10,13, 18,19
Y	WO,A,92 16221 (SYNERGEN INC) 1 October 1992 see page 10, line 18 - page 12, line 8; claims; example 17	1,4-8, 10,13, 18,19
A	DE,A,42 28 839 (BEHRINGWERKE AG) 3 March 1994 see claims; examples 1,2	1,19
A	EP,A,0 419 091 (SCHERING CORP) 27 March 1991 see page 2, line 12 - line 25; claims; examples	1,13-18
A	WO,A,93 11234 (SCHERING CORP) 10 June 1993 see page 1, line 7 - page 2, line 3; claims; examples see page 3, line 4 - line 8	1,13-18

INTERNATIONAL SEARCH REPORT

ternational application No.
PCT/US95/13101

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 13-17
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although these claims are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Int. ional Application No
PCT/US 95/13101

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